

Microreview

Entry of oomycete and fungal effectors into plant and animal host cells

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Summary

Fungal and oomycete pathogens cause many destructive diseases of plants and important diseases of humans and other animals. Fungal and oomycete plant pathogens secrete numerous effector proteins that can enter inside host cells to condition susceptibility. Until recently it has been unknown if these effectors enter via pathogen-encoded translocons or via pathogen-independent mechanisms. Here we review recent evidence that many fungal and oomycete effectors enter via receptor-mediated endocytosis, and can do so in the absence of the pathogen. Surprisingly, a large number of these effectors utilize cell surface phosphatidylinositol-3-phosphate (PI-3-P) as a receptor, a molecule previously known only inside cells. Binding of effectors to PI-3-P appears to be mediated by the cell entry motif RXLR in oomycetes, and by diverse RXLR-like variants in fungi. PI-3-P appears to be present on the surface of animal cells also, suggesting that it may mediate entry of effectors of fungal and oomycete animal pathogens, for example, RXLR effectors found in the oomycete fish pathogen, *Saprolegnia parasitica*. Reagents that can block PI-3-P-mediated entry have been identified, suggesting new therapeutic strategies.

Introduction

Oomycete and fungal pathogens

Oomycete and fungal pathogens cause a vast array of important diseases of plants, animals and humans. Oomycetes and fungi belong to distinct kingdoms of life,

but have convergently evolved many morphological and physiological similarities, including hyphae, absorptive heterotrophy and the ability to defeat plant and animal immune systems (Money, 1998; Latijnhouwers *et al.*, 2003). Some of the most devastating fungal diseases of plants include cereal rusts, rice blast, chestnut blight and Dutch elm disease (Agrios, 1988; Van Alfen, 2001). Some of the most important oomycete plant diseases include late blight of potato (caused the Irish potato famine), *Phytophthora* root rot of soybean, sudden oak death and downy mildews of maize (Agrios, 1988; Erwin and Ribiero, 1996). Some important fungal diseases of animals and humans include bat white nose syndrome, chytridiomycosis of amphibians, and candidiasis, coccidiomycosis, and cryptococcosis of humans (Ainsworth, 1973; Nucci and Marr, 2005). Oomycetes primarily cause diseases of aquatic animals such as fish, crustaceans and insect larvae, but also pythiosis of humans and other animals (Phillips *et al.*, 2008).

Plant defence mechanisms

Plant defences rely entirely on innate immunity since the cell walls of plant cells preclude motile professional immune cells (Jones and Dangl, 2006). Major defence mechanisms include constitutive and induced physical and chemical barriers, degradative enzymes, reactive oxygen species and programmed cell death (which is especially effective against obligate parasites). Diffusible and volatile signal molecules such as salicylate, methyl jasmonate and ethylene communicate danger signals throughout plant tissues and from plant to plant (Jones and Dangl, 2006). Cell surface pattern recognition receptors (PRRs) can detect commonly occurring pathogen molecules (pathogen-associated molecular patterns; PAMPs) such as flagellin from bacteria and chitin and glucan cell wall fragments from fungi and oomycetes. PRR recognition of PAMPs results in a set of responses called PAMP-triggered immunity (Jones and Dangl, 2006). Plants also produce large numbers of intracellular receptors containing nucleotide binding sites and leucine rich repeats (NB-LRR proteins) that can detect the presence of pathogen proteins that have entered the

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cytoplasm. Recognition of effector proteins by NB-LRR proteins triggers very vigorous and effective defence responses called effector-triggered immunity (Jones and Dangl, 2006). Because effector-triggered immunity can provide a highly effective defence, plant genes encoding NB-LRR proteins that recognize the presence of specific pathogen effectors are termed resistance (*R*) genes. Effectors recognized by NB-LRR proteins have historically been called 'avirulence' (*Avr*) proteins. Animal defences against fungal and oomycete pathogens rely heavily on innate as well as adaptive immune mechanisms (Romani, 2011). Animal innate immunity also involves detection of PAMPs by PRRs (Nurnberger *et al.*, 2004; Akira *et al.*, 2006). Animal immune mechanisms are generally effective, as most fungal pathogens of animals are opportunistic, causing disease mainly on immune-compromised hosts (Romani, 2011).

Effector proteins of plant and animal pathogens

A major weapon used against plant and animal immunity machinery by cellular pathogens, including bacteria, fungi and oomycetes, are secreted effector and toxin proteins that can enter into the cytoplasm of host cells (reviewed in Torto-Alalibo *et al.*, 2009; 2010). Effectors characterized to date, mostly from bacteria, target numerous host proteins associated with innate immunity including PRR signalling domains, MAP kinases, the host cytoskeleton, the host programmed cell death machinery, the host ubiquitination machinery, the host poly(ADP)-ribosylation machinery and the host transcriptional machinery (Torto-Alalibo *et al.*, 2009; 2010).

Cell entry by bacterial and protist effectors

Bacterial pathogens of plants and animals (and some mutualists also) utilize a variety of specialized secretion systems including type III, type IV and type VI machinery to inject proteins directly into the host cytoplasm, with the majority of effectors entering via the type III secretion machinery (Tseng *et al.*, 2009). An alternative strategy for cell entry is used by the class of bacterial effector proteins commonly referred to as toxins, such as cholera toxin, tetanus toxin and diphtheria toxin (Sandvig *et al.*, 2010). These proteins are released into the extracellular space via the type II secretion machinery, and then enter host cells via receptor-mediated endocytosis. In many cases, the proteins bind glycoproteins and/or glycolipids associated with lipid rafts and enter via lipid raft-mediated endocytosis. Once inside endosomes, the proteins escape into the cytosol by one of two general mechanisms (Sandvig and van Deurs, 2002; Sandvig *et al.*, 2010). In one mechanism, acidification of the endosome results in partial denaturation of the protein revealing

hydrophobic residues that allow crossing of the endosomal membrane (Sandvig and van Deurs, 2002; Sandvig *et al.*, 2010). In the other mechanism, the protein undergoes retrograde transport from the endosomes to the endoplasmic reticulum where it is translocated into the cytoplasm via a mechanism normally used to remove misfolded proteins (Sandvig and van Deurs, 2002; Sandvig *et al.*, 2010).

Apicomplexan parasites such as *Plasmodium* and *Toxoplasma* also use specialized machinery to transfer effector proteins into the cytoplasm of host cells (Boothroyd and Dubremetz, 2008; de Koning-Ward *et al.*, 2009). *Plasmodium* uses a translocon to transport effector proteins from the lumen of the parasitophorous vacuole into the cytoplasm of the erythrocyte; the transferred proteins carry a motif required for cell entry called Pexel (RxLx^F/D/Δ) that is cleaved and acetylated during the transfer process (de Koning-Ward *et al.*, 2009). *Toxoplasma* and several other apicomplexan parasites use a specialized secretory organelle called a rhoptry to directly inject effector proteins into host cells immediately prior to invasion of the cell by the parasite (Boothroyd and Dubremetz, 2008).

Cell entry by fungal and oomycete effectors

Until recently, relatively little was known about the mechanisms by which effector proteins from oomycetes and fungi could enter host cells. That entry could occur was inferred from the fact that many plant *R* genes conferring resistance against fungal and oomycete pathogens turned out to encode intracellular NB-LRR proteins (Tyler, 2002; Ellis *et al.*, 2006). Furthermore, many of the pathogen proteins recognized by those *R* gene products proved to be small secreted proteins, indicating that a cell entry mechanism must exist (Ellis *et al.*, 2006; Tyler, 2009). Among fungal and oomycete animal pathogens, there was little evidence that any pathogen proteins could enter the host cytoplasm, with the exception of some fungal ribotoxins (Lacadena *et al.*, 2007). Here we review recent discoveries regarding the mechanism of entry of oomycete and fungal proteins into plant cells, and evidence that similar mechanisms might operate during animal infections.

Oomycete and fungal plant pathogen effectors that enter cells autonomously

Many oomycete and fungal effectors have been shown to directly enter host cells while others are inferred to enter based on their intracellular interactions (Tyler, 2011). Direct entry can be visualized using an attached fluorescent tag or by a reporter protein that induces cell death when in the cytoplasm (e.g. a characterized effector that

interacts with an intracellular NB-LRR proteins). Several avirulence effectors have been inferred to enter cells based on their physical interaction with host NB-LRR *R* proteins. Examples include the fungal effectors AvrL567, AvrM, and Avr-Pita, and the oomycete effectors ATR1 and Avr3a (Jia *et al.*, 2000; Dodds *et al.*, 2006; Kanzaki *et al.*, 2008; Catanzariti *et al.*, 2010; Krasileva *et al.*, 2010). Many other proteins have been inferred to translocate into cells based on their physiological effects. For example, *P. infestans* CRN2, CRN8, CRN16, and *P. sojae* PsCRN63, all activate programmed cell death when expressed inside cells (Haas *et al.*, 2009; Schornack *et al.*, 2010; Liu *et al.*, 2011). Avr1a, Avr1b, Avh331, SNE1, PsCRN115, PexRD8, PexRD35₄₅₋₁ and AvrPiz-t suppress different inducers of cell death when expressed intracellularly (Dou *et al.*, 2008a; Oh *et al.*, 2009; Qutob *et al.*, 2009; Kelley *et al.*, 2010; Liu *et al.*, 2011).

Oomycete and fungal effector cell entry motifs

Oomycete effector RXLR and dEER motifs

The RXLR and dEER motifs were originally hypothesized to constitute a cell entry domain due to their conservation among four known oomycete avirulence proteins (Rehmany *et al.*, 2005; Birch *et al.*, 2006). The oomycete avirulence proteins were thought to translocate into host cells because of the presumed intracellular localization of their respective *R* gene products.

The role of the RXLR motif in cell entry was initially verified by stable transformation of *P. infestans* and *P. sojae* with the *Avr3a* and *Avr1b* genes respectively (Whisson *et al.*, 2007; Dou *et al.*, 2008b). RXLR and dEER motif mutations in the transgenes resulted in the loss of resistance mediated by the respective *R* genes *R3a* and *Rps1b*. Cytoplasmic expression of these mutant proteins in the host plant cytoplasm still resulted in *R* gene-mediated cell death, demonstrating that the mutations were interfering with translocation and there was not simply a loss of recognition by the particular *R* protein. A chimeric protein containing the N-terminal RXLR-dEER domain of *Avr3a* fused to the enzyme beta-glucuronidase was transported into potato cells from stable *P. infestans* transformants, indicating that the domain was sufficient for cell entry (Whisson *et al.*, 2007).

Dou *et al.* (2008b) showed that the RXLR-dEER domain of *Avr1b* could translocate both the full-length effector protein and GFP into a variety of plant cell types in the absence of any pathogen-encoded machinery. Using a novel double barrel particle bombardment assay the RXLR-dEER domains of *P. sojae* effectors *Avr1b*, *Avh5* and *Avh331* were expressed as fusion proteins to the C-terminus of *Avr1b*, and secreted from soybean leaf cells (Dou *et al.*, 2008b; Kale *et al.*, 2010). Re-entry was

measured by programmed cell death induced by the interaction of *Rps1b* and the C-terminus of *Avr1b*. Mutations in the RXLR motifs abolished re-entry (Dou *et al.*, 2008b). In another assay, the RXLR-dEER domains of *Avr1b*, *Avh5* or *Avh331* were coupled to the N-terminus of GFP and then the purified fusion proteins were incubated with soybean root cells (Dou *et al.*, 2008b; Kale *et al.*, 2010). Entry into root cells occurred for all three RXLR-dEER fusion proteins, while proteins with mutations of the RXLR or dEER motifs did not enter cells (Dou *et al.*, 2008b; Kale *et al.*, 2010).

Oomycete genomes contain numerous genes encoding RXLR-dEER proteins. A methodical search using hidden markov models resulted in the identification of 396 and 374 RXLR-dEER effector gene respectively from the genomes of *P. sojae* and *P. ramorum* (Jiang *et al.*, 2008), while 563 and 134 putative RXLR genes were identified in the *P. infestans* (Haas *et al.*, 2009) and *H. arabidopsidis* (Baxter *et al.*, 2010) genomes respectively.

RXLR-like motifs in fungal plant pathogen effectors

The hidden markov model generated for the oomycete RXLR motif is unable to identify RXLR motifs in fungal effectors. Furthermore, bioinformatic analysis of fungal effectors and genomes has not resulted in the identification of any apparent conserved motifs comparable to the RXLR motif (Catanzariti *et al.*, 2006), with the possible exception of a candidate motif in the genomes of powdery mildew fungi (Godfrey *et al.*, 2010). As an alternative approach to finding fungal cell entry motifs, Kale *et al.* (2010) performed detailed functional mutagenesis experiments of the *P. sojae* *Avr1b* RXLR motif to identify which residues of the motif are functionally flexible. The arginine at position 1 could be replaced with lysine (K) or histidine (H) and retain translocation activity. The leucine at position 3 could be replaced by large, hydrophobic amino acids including Methionine (M), Phenylalanine (F), Tyrosine (Y), Tryptophan (W) and Isoleucine (I), but not by alanine (A) or valine (V). At position 4 the arginine could be replaced with a diversity of amino acids including glycine and retain translocation activity. From these results, a more flexible 'RXLR-like' motif [R,K,H] × [L,I,M,F,Y,W] was defined (Kale *et al.*, 2010).

The extended RXLR-like motif successfully identified functional cell entry motifs in fungal effectors known to localize inside the host plant cell. RXLR-like cell entry motifs were identified in *Melampsora lini* AvrL567 (RFYR), *Fusarium oxysporum* Avr2 (RIYER) and *Leptosphaeria maculans* AvrLm6 (RYWT) (Kale *et al.*, 2010). The RFYR motif of AvrL567 was independently shown to mediate translocation (Rafiqi *et al.*, 2010), and the same study identified three putative RXLR-motifs within the translocation domain of another *M. lini* effector AvrM.

Subsequently, a secreted effector of the mutualistic ectomycorrhizal fungus *Laccaria bicolor*, MiSSP7, was shown to be translocated into poplar and soybean cells through its RXLR-like motif, RALG (Plett *et al.*, 2011). MiSSP7 is the first example of a beneficial fungal interaction utilizing RXLR-like protein translocation.

Some oomycete effectors may also utilize RXLR-like motifs rather than strict RXLR motifs, for example, ATR5 from *H. arabidopsidis* (Bailey *et al.*, 2011) and QXLR-containing proteins predicted from the genome of *Pseudoperonospora cubensis* (Tian *et al.*, 2011).

Translocation mediated by the RGD motif

The protein toxin ToxA is produced by *Pyrenophora tritici-repentis* and *Stagonospora nodorum*, two fungal pathogens of wheat (Liu *et al.*, 2006; Ciuffetti *et al.*, 2010). ToxA causes cell death when expressed inside wheat cells. Extracellular application of ToxA also induced cell death symptoms similar to the pathogen induced disease symptoms. ToxA cell entry was confirmed by the use of fluorescently tagged ToxA and a protease assay (Manning and Ciuffetti, 2005). The crystal structure of ToxA contains an exposed loop of 10 amino acids (Sarma *et al.*, 2005), of which 6 match the RGD (arginine-glycine-aspartate) loop of the animal extracellular protein vitronectin. The ToxA RGD motif bound with high affinity to sites on the plant cell membrane, and was required for entry into plant cells (Manning *et al.*, 2008); thus it was suggested that the entry mechanism may be receptor-mediated endocytosis (Manning *et al.*, 2008). In animals, several extracellular adhesive proteins, including vitronectin, bind a common cell surface receptor, integrin, through the RGD motif, and a number of integrin-binding ligands enter cells by caveolae-mediated endocytosis. Although RGD receptors were not identified in the ToxA system, lectin-like receptor kinases capable of binding RGD-containing peptides were identified in *Arabidopsis* (Gouget *et al.*, 2006). Some oomycete RXLR effectors also contain RGD motifs capable of binding them to plant membranes (Senchou *et al.*, 2004), but it is currently unknown if the RGD motifs can also mediate cell entry.

Translocation mediated by oomycete Crinkler motifs

The large and diverse Crinkler (CRN) family of proteins is found in diverse oomycete genomes (Torto *et al.*, 2003; Tyler *et al.*, 2006; Gaulin *et al.*, 2008; Haas *et al.*, 2009; Levesque *et al.*, 2010). The CRN proteins were first identified via high throughput screens of putative secreted proteins expressed during *P. infestans* infection (Torto *et al.*, 2003). Overexpression of the CRN proteins in leaves resulted in a crinkling and cell-death-phenotype along with activation of several defence related genes

(Torto *et al.*, 2003). Proteins in the CRN super-family consist of a conserved 50–60 amino acid N-terminus commonly referred to as the LFLAK domain and divergent C-terminal domains (Torto *et al.*, 2003; Haas *et al.*, 2009). A number of these C-terminal domains resemble host intracellular proteins, suggesting intracellular targets for the CRN effectors (Haas *et al.*, 2009). In the presence or absence of the secretory leader sequence expression of certain CRN proteins *in planta* resulted in either cell death or suppression of cell death (Haas *et al.*, 2009; Liu *et al.*, 2011). These results suggested that CRN proteins may also have the ability to translocate into host cells.

When the LFLAK domains from three *P. infestans* Crinkler proteins (CRN3, CRN8 and CRN16) or from *Aphanomyces euteiches* CRN AeCRN5 were fused to the C-terminal domain of *P. infestans* Avr3a in place of the RXLR translocation domain, the LFLAK domain mediated translocation of the CRN–Avr3a fusion protein into *N. benthamiana* leaf cells from *P. capsici* transformants during infection (Schornack *et al.*, 2010). These findings indicate that the LFLAK domain can complement the cell entry function of the absent RXLR domain. Mutations of the namesake LFLAK motif indicated the motif is critical for the cell entry activity of the CRN–Avr3a fusion protein.

Translocation associated with CHXC motifs

A recent analysis of the genome of the obligate oomycete pathogen *Albugo laibachii* (Kemen *et al.*, 2011) identified a class of predicted secreted proteins with a statistically significant overabundance of the sequence motif CHxC (cysteine, histidine, any, cysteine) in their N-terminus. The N-terminal domain of one CHxC protein, CHXC9, could carry the C-terminus of *P. infestans* Avr3a into *N. benthamiana* cells when expressed in *P. capsici* (Kemen *et al.*, 2011). Mutation of the CHxC motif to AAAA (four alanines) eliminated most of the translocation activity, suggesting that the motif played an important role in the function of the translocation domain (Kemen *et al.*, 2011). Intriguingly, two RXLR-like motifs, KYLG and RLYW, lie very close to the CHxC motif in CHXC9; it will be interesting to test if either of these motifs also contributes to entry by this putative effector.

Additional putative entry motifs in fungal and oomycete effectors

Several potential translocation motifs have been identified based on alignment of putative secreted proteins from a given genome or by homology among effectors inferred to enter cells. So far however, there is no experimental evidence suggesting these motifs play a role in protein translocation. A [L/I]xAR motif was identified in several rice blast effectors (Yoshida *et al.*, 2009). A family of putative

secreted effectors from *Pythium ultimum* share a YxSL[R/K] motif (Levesque *et al.*, 2010). Interestingly, *Blumeria graminis* f. sp. *hordei* AVRk1 and AVRa10 share a [R/K]VY[L/I]R motif (Ridout *et al.*, 2006) that resembles the RXLR-like motifs identified by (Kale *et al.*, 2010). A novel motif [Y,F,W]xC is conserved in many candidate effectors from powdery mildew and rust fungi (Godfrey *et al.*, 2010).

Effector entry via the fungal biotrophic interfacial complex

Magnaporthe oryzae, the rice blast fungus, is a hemi-biotrophic pathogen of rice. *M. oryzae* directly penetrates rice epidermal cells through an appressorium. An extra-invasive-hyphal membrane (EIHM) encapsulates the invading hyphae and continues to surround hyphae spreading to adjacent cells (Kankanala *et al.*, 2007). Effectors translocating into host cells must cross both the EIHM and host cell plasma membrane. Nonetheless, there are at least six *M. oryzae* effectors that translocate into host cells. Several of these effectors are avirulence proteins that are recognized by intracellular NBS-LRR proteins (Jia *et al.*, 2000; Orbach *et al.*, 2000; Li *et al.*, 2009; Yoshida *et al.*, 2009). Fluorescent tagging of two *M. oryzae* secreted effectors, Bas1 and Pwl2, allowed direct imaging of the translocated effectors into host cells (Khang *et al.*, 2010). A novel structure, termed the biotrophic interfacial complex (BIC), forms upon penetration of a host cell (Khang *et al.*, 2010). Intracellular effectors such as Avr-Pita, Pwl2 and BAS1 all accumulate in the BIC, while extracellular effectors such as BAS4 do not accumulate in the BIC (Khang *et al.*, 2010). These findings suggest that the BIC is a specialized structure mediating the delivery of effectors into rice cells. However, the molecular mechanisms that effectors might use for BIC translocation into host cells are currently unknown, and no highly conserved motif such as RXLR has been experimentally validated.

Pathogen-independent entry into plant and animal cells by oomycete and fungal effectors

There has been considerable debate whether oomycete and fungal effectors enter plant cells via specialized machinery such as the bacterial type III secretion system or the *Plasmodium* Pexel translocon, or whether they enter by receptor-mediated endocytosis independently of the pathogen like many of the bacterial toxins (e.g. Ellis *et al.*, 2006; Birch *et al.*, 2008). Similarities between the *Plasmodium* Pexel motif and the oomycete RXLR motif encouraged early speculation that oomycetes might use a translocon (Bhattacharjee *et al.*, 2006). However, as summarized below, the weight of experimental evidence now

supports that entry occurs independently of the pathogen, probably via receptor-mediated endocytosis. Most of the studies involved effectors from plant pathogens and entry into plant cells. However, entry of RXLR(-like) effectors into animal cells has also been observed.

A variety of assays (Fig. 1) have been utilized to demonstrate pathogen-independent entry by oomycete and fungal effectors (Dou *et al.*, 2008b; Kale *et al.*, 2010; Rafiqi *et al.*, 2010; van West *et al.*, 2010; Plett *et al.*, 2011). Using C-terminal fusions to fluorescent proteins, full-length proteins or N-termini of oomycete effectors Avr1b, Avh5 and Avh331, and of fungal effectors AvrL567, Avr2, AvrLm6, AvrM and MiSSP7 were directly shown to enter cells in the absence of any pathogen-encoded machinery (Dou *et al.*, 2008b; Kale *et al.*, 2010; Rafiqi *et al.*, 2010; Plett *et al.*, 2011). Fluorescent antibodies were utilized to show that the putative RXLR effector SpHTP1 from the fish pathogen *Saprolegnia parasitica* could enter fish cells in the absence of the pathogen (van West *et al.*, 2010). In the case of entry into poplar root cells by the mutualism effector MiSSP7, a fluorescent chromophore was conjugated to the protein (Plett *et al.*, 2011). When infiltrated into soybean leaves purified full-length Avr1b and Avh331 proteins triggered strong defence-related cell death in the presence of the *Rps1b* and *Rps1k* resistance genes respectively. *Rps1k* encodes an NB-LRR protein and *Rps1b* is an allele of *Rps1k*; therefore, induction of cell death is taken as evidence of effector translocation into the host cells. Mutation of the RXLR and RXLR-like motif for all the above mentioned effectors resulted in a loss of effector translocation into host cells.

Transient expression of effectors *in planta* via particle bombardment transformation or *Agrobacterium*-mediated transformation has also been utilized to study effector cell entry in the absence of the natural pathogen (Fig. 1). The effectors are designed to be secreted from the plant cells and then tested if they can translocate back into the cells. Transient expression via particle bombardment has been utilized to identify the translocation motif of Avr1b, Avh5, Avh331, AvrL567, Avr2 and AvrLm6 (Dou *et al.*, 2008b; Kale *et al.*, 2010). In each case the translocation domain was fused to the C-terminal domain of Avr1b and re-entry was measured by the cell death triggered by Avr1b in the presence of *Rps1b*. Transient expression via *Agrobacterium* has been utilized to identify and characterize the RXLR-like motifs from AvrL567 and AvrM required for re-entry into flax or tobacco cells (Rafiqi *et al.*, 2010).

Effector entry into animal cells has been demonstrated in two contexts. In one, as mentioned above, an oomycete effector, SpHTP1, from the fish pathogen *S. parasitica* could enter fish cells in the absence of the pathogen (van West *et al.*, 2010). In the second context Kale *et al.* (2010) demonstrated that effectors from oomycete and fungal

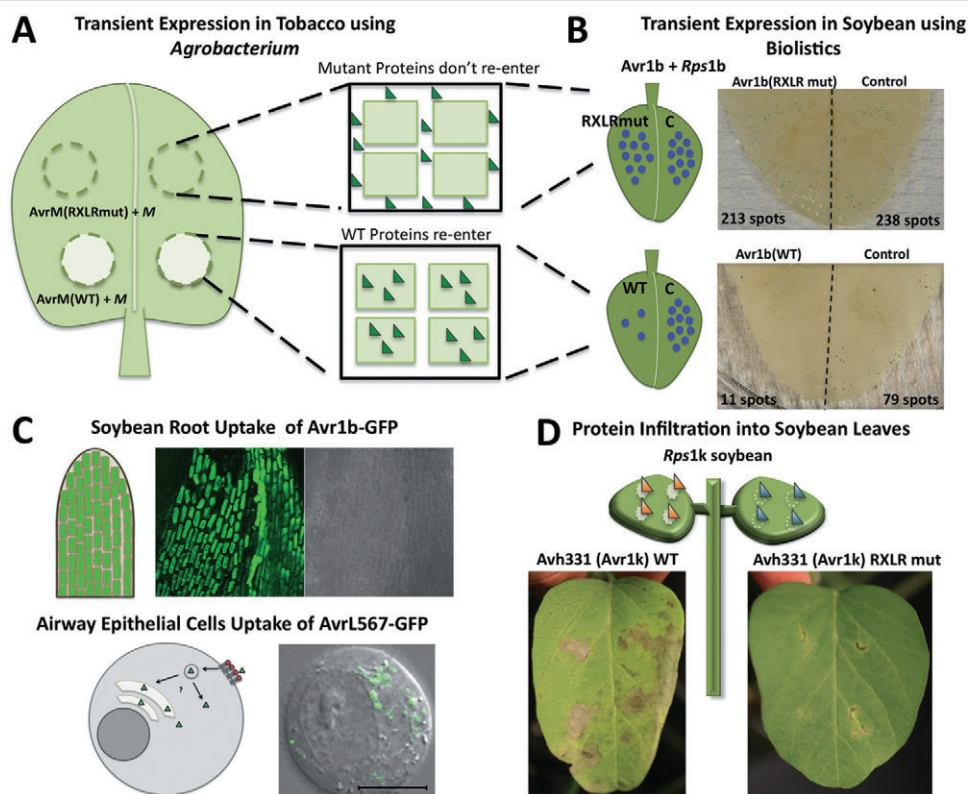


Fig. 1. Assays for detecting pathogen-independent entry of effectors.

A and B. Effectors are transiently expressed *in planta* using either *Agrobacterium tumefaciens* infiltration (A) or particle bombardment (B). In both assay systems, a secretory leader directs the effector outside the cell; cell entering effectors then translocate back into the plant cells where they may interact with a resistance protein leading to an induction of cell death. RXLR and RXLR-like mutants are no longer capable of cell re-entry and localize in the apoplastic spaces. Translocation is reported either by a fluorescent reporter such as GFP or an induction of cell death by a resistance protein. (A) T-DNA containing a plant gene expression cassette encoding the effector is transferred into tobacco cells by *A. tumefaciens*. Effector re-entry results in tissue-wide cell death. (B) Tungsten particles are coated with plasmids encoding an effector and the cell vitality reporter beta-glucuronidase, which produces a blue pigment in living cells when appropriately stained. Bombardment with the particles physically inserts the plasmids into cells. Cell death resulting from effector re-entry produces a reduction of blue spots. C. Purified effector–GFP fusion protein is incubated with soybean roots or human airway epithelial cells. Soybean roots are thoroughly washed to remove uninternalized protein localized to apoplastic spaces. Effector–GFP fusion can be seen entering airway epithelial cells after short exposure (less than 5 min). RXLR and RXLR-like mutants are unable to enter cells. D. Purified effector protein (Avh331) is infiltrated into soybean leaves expressing *Rps1k*. Internalization of Avh331 leads to induction of tissue-wide cell death based on the interaction with *Rps1k*. RXLR mutants are unable to enter cells and therefore do not induce tissue-wide cell death.

plant pathogens could enter human lung epithelial cells, suggesting that the same host cell machinery for effector entry may be present in human cells as in plant cells. Thus potentially oomycete and fungal pathogens of humans and other animals might utilize RXLR-like effectors. However, other than in *S. parasitica*, this possibility has not yet been tested.

Based on the location of internalized effector proteins in vesicle-like structures and results from a spectrum of endocytosis inhibitors, it appears likely that effector entry into both plant and animal cells involves lipid-raft-mediated endocytosis (Kale *et al.*, 2010; Rafiqi *et al.*, 2010; Plett *et al.*, 2011) (Fig. 2).

Pathogen-independent entry by effectors is important during infection as it suggests that effectors could travel through the intercellular spaces of host tissue to enter

host cells and condition them for susceptibility in advance of pathogen hyphae (Fig. 2). In *P. sojae* many effector genes are expressed before the pathogen contacts the plant (Wang *et al.*, 2011), and Shan *et al.* (2004) suggested that *P. sojae* Avr1b could spread throughout an entire soybean plant.

Endocytosis of oomycete and fungal effectors into plant and animal cells via binding to external phosphatidylinositol-3-phosphate

Although numerous bacterial and plant toxin proteins utilize glycoprotein or glycosphingolipid receptors to mediate entry by endocytosis (Sandvig *et al.*, 2010), current evidence indicates that a variety of oomycete and fungal RXLR(-like) effectors may utilize a lipid receptor

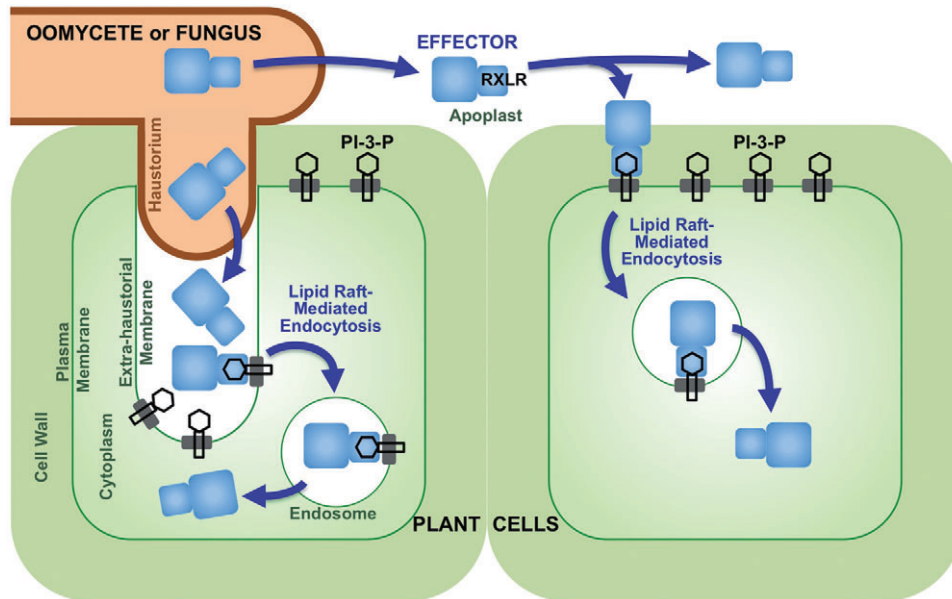


Fig. 2. Model for entry of fungal and oomycete effectors into plant cells. Two routes of entry appear likely, directly from the apoplast (extracellular space), possibly far in advance of the pathogen hyphae, and from the haustorium (a hyphae specialized for nutrient uptake that pierces the cell wall and invaginates the plasma membrane). In both cases, binding to cell surface phosphatidylinositol-3-phosphate (PI-3-P) via the RXLR domain precedes entry via lipid-raft-mediated endocytosis. The mechanism by which the effectors escape the endosomes is currently unknown. Fungal and oomycete effectors may enter animal cells by the same mechanism.

previously known only as an intracellular molecule, namely phosphatidylinositol-3-phosphate (PI-3-P) (Kale *et al.*, 2010; Plett *et al.*, 2011) (Fig. 2). Kale *et al.* (2010) showed that the oomycete effectors Avr1b, Avh5, Avh331 and the fungal effectors AvrL567, Avr2 and AvrLm6 bound to phosphatidylinositol-3-phosphate (PI-3-P) and in several cases also to PI-4-P. In all cases, the same RXLR or RXLR-like motifs that were required for cell entry were also required for PI-3-P binding, while RXLR and RXLR-like motifs not required for cell entry were not required for PI-3-P binding (Kale *et al.*, 2010). Furthermore, using specific binding proteins, Kale *et al.* (2010) showed that PI-3-P (but not PI-4-P) could be detected on the surface of plant and animal cells, and that entry of the effectors could be blocked by the PI-3-P-binding proteins or by the competitive inhibitor inositol-1,3-bisphosphate. Plett *et al.* (2011) showed the fungal mutualism effector, MiSSP7, also bound to PI-3-P via its cell entry motif RALG, and that entry into poplar root cells could be blocked by PI-3-P-binding proteins or inositol bisphosphate.

Although PI-3-P is well characterized as an intracellular lipid involved in vesicle trafficking and autophagy, its presence on the outside of cells was surprising. Several other examples exist of internal acidic phospholipids appearing on the outside of cells, including PI-4-P released by plant cells (Gonorazky *et al.*, 2008; 2011; Regente *et al.*, 2008), phosphatidylinositol-3,4,5-triphosphate on the surface of *Neisseria*-infected human cells (Lee *et al.*, 2005) and non-apoptotic externalization of phosphatidylserine during

infection of human cells by *Chlamydia* (Goth and Stephens, 2001) and by *Helicobacter* (Murata-Kamiya *et al.*, 2010). However, the presence of external PI-3-P remains somewhat controversial, and further work to confirm its presence chemically, and to establish the mechanisms by which it may reach the outer leaflet is needed.

Concluding remarks

The weight of evidence currently supports the hypothesis that entry of diverse families of oomycete and fungal effectors into host cells resembles the entry of bacterial and plant toxin proteins via receptor-mediated endocytosis (Fig. 2), rather than via pathogen-encoded injectisomes or translocons. Surprisingly, several oomycete and fungal effectors appear to have independently evolved to utilize a lipid receptor previously unknown to be on the cell surface, namely PI-3-P (Fig. 2). Even more surprisingly, PI-3-P appears to occur on the surface of at least some animal cells, leading to the speculation that fungal and oomycete pathogens of animals might also utilize effectors that enter host cells via PI-3-P binding. It is not however ruled out that some fungal or oomycete effectors, for example CRN, CHXC or [Y,F,W]xC effectors, might have evolved to bind other lipids, or that some RXLR(-like) effectors might bind a wider range of lipids than just PI-3-P. Since PI-3-P-binding appears to have evolved independently at least twice (in oomycetes and

fungi) and perhaps multiple times within each kingdom, it seems plausible that PI-3-P-binding effectors might have also evolved in other groups of pathogens and pests such as bacteria, protists, nematodes or insects. The presence of PI-3-P on host cell surfaces also predicts that PI-3-P is utilized for trafficking of host proteins, but this has yet to be tested. Finally, the ability to block PI-3-P-mediated effector entry (Kale *et al.*, 2010) suggests promising avenues for disease control.

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